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Original Article

NADPH oxidase-derived superoxide anion mediates angiotensin II-induced cardiac hypertrophy

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Abstract

Cardiac hypertrophy is an adaptive response to increases in blood pressure. Recent studies indicate that the hypertrophic process is associated with increases in intracellular oxidative stress in cardiomyocytes. We hypothesize that superoxide anion mediates the hypertrophic response and that antioxidant therapy may be effective in attenuating cardiac hypertrophy. Neonatal rat cardiac myocytes were stimulated with angiotensin II (AngII, 1 μM) with and without various antioxidants. *N*-acetylcysteine (NAC, 10 mM) and probucol (50 μM), and to a lesser extent, vitamin C (500 μM) and reduced glutathione (1 mM), inhibited AngII-induced [^{3}H]-leucine uptake and atrial natriuretic factor (ANF) promoter activity. The hypertrophic response is mediated by superoxide anion ($O_2^{-}\cdot$) since cell-permeable polyethylene glycol (PEG)-conjugated superoxide dismutase (50 U/ml), but not PEG-catalase (500 U/ml), attenuated AngII-induced [^{3}H]-leucine uptake and ANF promoter activity. Furthermore, NAC blocked AngII-induced increase in myocardial oxidative stress, decreased the expression of ANF and myosin light chain-2v, and inhibited the re-organization of cytoskeletal proteins, desmin and α-actinin. These effects of AngII were abolished by angiotensin type 1 receptor blocker, losartan, but not type 2 receptor blocker, PD123319. Indeed, co-administration of losartan (10 mg/kg/d, 14 d) or NAC (200 mg/kg/d, 14 d) inhibited AngII-induced $O_2^{-}\cdot$ production and cardiac hypertrophy in rats without affecting blood pressure. These findings indicate that the generation of $O_2^{-}\cdot$ contributes to oxidant-induced hypertrophic response and suggest that antioxidant therapy may have beneficial effects in cardiac hypertrophy.

Keywords: Antioxidants; Cardiomyocyte; Hypertrophy; Angiotensin II; Reactive oxygen species; G-proteins; NADPH oxidase

1. Introduction

Cardiac hypertrophy is a physiological adaptive response to hypertension and increased afterload [1]. However, cardiac hypertrophy often carries a poor prognosis because of an increased risk of arrhythmia [2] and the development of congestive heart failure [3]. Recent studies suggest that increased production of reactive oxygen species (ROS) is involved in the hypertrophic process. For example, ROS increase the expression of proto-oncogenes, such as *c-myc* and *c-fos* [4], mediate the linkage of Na^+/K^+ ATPase to hypertrophy, and activate mitogen-activated protein kinase (MAPK) [5]. Treatment with antioxidants inhibits the hypertrophic response of cardiac myocytes [6,7]. Various vasoactive peptides, such as angiotensin II (AngII) and endothelin-1, play a pivotal role in the induction of these hypertrophic responses. Stimulation of the AngII type 1 receptor (AT1R) in cardiac myocytes induces a hypertrophic response mediated, in part, by the activation of small GTP-binding proteins belonging to the Rho GTPase family.

We have recently shown that the HMG-CoA reductase inhibitors or statins prevent the development of cardiac hypertrophy through a novel antioxidant mechanism involving inhibition of Rac1 geranylgeranylation [8]. The small GTP-binding protein, Rac1, is known to regulate the production of superoxide anions in various tissues [8,9]. However, it is not known which ROS is associated with the development of

Abbreviations: AngII, angiotensin II; AT1R, angiotensin II type 1 receptors; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; GSH, glutathione; VitC, vitamin C; ANF, atrial natriuretic factor; MLC, myosin light chain; PBS, phosphate buffer saline; LV, left ventricle; BW, body weight; NADH, β-nicotinamide adenine dinucleotide; NADPH, β-nicotinamide adenine dinucleotide phosphate.

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cardiac hypertrophy. The purpose of this study, therefore, was to determine which ROS contribute to the development of cardiac hypertrophy and whether antioxidant therapy could directly attenuate the hypertrophic process.

2. Materials and methods

2.1. Reagents

All tissue culture reagents were purchased from Life Technologies Gibco BRL (Grand Island, NY). [^{3}H]-leucine and [^{35}S]-GTPγS was purchased from New England Nuclear-Life Science Products (Boston, MA). Penicillin and streptomycin were obtained from BioWhittaker (Walkersville, MD). All other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO). Anti-cardiac myosin heavy chain (MHC) antibody was obtained from Biogenesis (Sandown, NH). Collagenase type 2 was purchased from Worthington (Lakewood, NJ). 2',7'-dichlorofluorescin (DCF)-diacetate was obtained from Molecular Probes Inc. (Eugene, OR). The atrial natriuretic factor (ANF) cDNA and promoter-luciferase reporter construct were kindly provided by Joan Heller Brown (University of California, San Diego, CA). Losartan was obtained from Merck Research Laboratories (Rahway, NJ), and PD123319 was obtained from Research Biochemicals International (Natick, MA).

2.2. Cardiac myocyte culture

Cardiac myocytes were obtained from ventricles of 1-d-old Spraque-Dawley rats and isolated and cultured as described [8]. Using this method, >95% of the cells were cardiac myocytes as assessed by immunofluorescence staining with an anti-cardiac MHC antibody. Viability was determined by cell number, frequency of contractions (i.e. intrinsic heart rate), cellular morphology, and trypan blue exclusion.

2.3. Measurements of cardiac hypertrophy

Cardiac myocyte hypertrophy was determined by [^{3}H]-leucine uptake as described [8]. After 36 h in serum-free insulin-transferrin (IT) medium, cardiac myocytes were treated with the indicated conditions in the presence of diluents or [^{3}H]-leucine (1 μCi/ml) for 24 h. After incubating at room temperature for 45 min, cellular proteins were precipitated with 5% TCA, resuspended in 0.4 N NaOH, and the radioactivity was counted in a scintillation counter (Beckman LS 6000IC). ANF-Luc reporter plasmid, β-galactosidase and/or c-myc-tagged N17Rac1 were transfected in rat neonatal cardiomyocytes using the calcium-phosphate co-precipitation method [8]. Luciferase activity was normalized to β-galactosidase activity for each sample, to correct for variations in transfection efficiency. Results are expressed as relative ratio of control (fold induction).

2.4. Northern blotting

Equal amount of total RNA (15 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as previously described [8]. The ANF and myosin light chain (MLC)-2v cDNAs were labeled with random hexamer priming using [α-^{32}P]CTP (NEN Life Science Products) and Klenow (Pharmacia). Blots were analyzed by laser densitometry.

2.5. Immunofluorescence

Cells were incubated with a mouse monoclonal antibody to α-actinin (1:200 in 1% BSA) and a rabbit polyclonal antibody to desmin (Sigma Chemical Co., St. Louis, MO, 1:100 in 1% BSA). After washing with 1% BSA in phosphate buffered saline (PBS), FITC-conjugated goat anti-mouse IgG (H+L) (green fluorescence) and TRITC-conjugated anti-rabbit IgG (H+L) (red fluorescence) (Molecular Probes, Inc., Eugene, OR) were used as secondary antibodies (1:200 in 1% BSA). Immunofluorescence was visualized using a Bio-Rad MRC-1024/2P multiphoton microscope equipped with krypton–argon and Ti-sapphire lasers. Photographic images were taken from five random fields. Fluorescent intensities from randomly selected fields (around 50 cells per groups) were quantified with image-analyzing software (NIH image).

2.6. Rho GTP-binding activity

Membrane-associated Rho GTP-binding activity was measured in cultured cardiac myocytes or rat hearts by immunoprecipitating [^{35}S]-GTPγS-labeled RhoA or Rac1 using partially-purified cardiac myocyte membranes and specific RhoA and Rac1 antibodies (Santa Cruz Biotech., Santa Cruz, CA) as described [8].

2.7. Fluorescence microscopy study of intracellular oxidation

Rat neonatal cardiomyocytes were cultured on coverslips in 35 mm dishes. Intracellular generation of ROS was quantified using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as described [8]. Upon oxidation, DCFH is converted to DCF, a fluorescent compound. After stimulation, the cells were incubated 30 min with 10 μM DCFH-DA and immunofluorescence was visualized using a Bio-Rad MRC-1024/2P multiphoton microscope equipped with krypton-argon and Ti-sapphire lasers. In some experiments, cells were also co-stained with mouse anti-human c-myc antibody (1:100 in 1% BSA; Transduction Laboratories, Lexington, KY) followed by treatment with R-phycoerythrin-conjugated goat antibody (red fluorescence) as a second antibody (1:100 in 1% BSA). Photographic images were taken from five random fields.

2.8. Measurements of NADPH oxidase activity

We measured superoxide anion ($O_2^{-}\cdot$) production in cardiac myocytes or heart by monitoring the chemilumines-

cence (Berthold, Lumat LB9501) for 10 min in modified HEPES buffer (NaCl 140 mM, KCl 5 mM, $MgCl_2$ 0.8 mM, $CaCl_2$ 1.8 mM, Na_2HPO_4 1 mM, HEPES 25 mM, and 1% glucose, pH 7.2), containing 100 μg myocyte extract, 1 mM EGTA, and 5 μM lucigenin (final volume, 0.25 ml) as described [10]. The β-nicotinamide adenine dinucleotide phosphate (NADPH) or β-nicotinamide adenine dinucleotide (NADH) oxidase activity was measured with 100 μM NADPH or NADH as substrate. The results are expressed as relative light units (RLU) per microgram of cell extract.

2.9. Ferricytochrome c reduction assay

Assay for superoxide anion ($O_2^{-}\cdot$) released into the supernatant was carried out by measuring superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described [11]. After 24 h stimulation in phenol red-free and serum-deficient IT medium, ferricytochrome *c* was added to the supernatant to a final concentration of 70 μM in the presence or absence of SOD (100 U/ml). Reduction of ferricytochrome *c* in the supernatant was monitored for 10 min at an absorbance of 550 nm using a spectrophotometer (Spectra Max plus 384, Molecular Devices, Sunnyvale, CA). Rates of $O_2^{-}\cdot$ production were calculated as described [12]. The results are expressed as nmol/24 h/million cells. Intact, non-homogenized pieces of rat or mouse hearts (100–200 mg) were suspended in a Krebs bicarbonate buffer containing the following (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.1, KH_2PO_4 1.2, glucose 5.6, and $NaHCO_3$ 25, adjusted to pH 7.4 in tissue bath gassed with 21% O_2 and 5% CO_2 for 4 h. The resulting supernatant was used for measuring SOD-inhibitable reduction of ferricytochrome *c* as described [11]. The results were expressed as nmol of $O_2^{-}\cdot$ produced per gram of cardiac tissue.

2.10. Aconitase activity assay

The production of intracellular $O_2^{-}\cdot$ was determined indirectly by changes in aconitase activity [8]. Rat heart tissues were homogenized and resuspended in a buffer containing Tris–HCl (50 mM, pH 7.6), cysteine (1 mM), citrate (1 mM), and $MnCl_2$ (0.5 mM). Cellular extracts (15 μg of protein) were added to a reaction buffer (0.2 ml) containing Tris–HCl (50 mM, pH 7.4), isocitrate (20 mM), and $MnCl_2$ (0.5 mM) at 25 °C, and the formation of *cis*-aconitate from isocitrate was measured spectrophotometrically after 2 min at an absorbance of 240 nm. Aconitase activity was calculated using the extinction coefficient of 3.6/mM/cm and expressed as nmol of *cis*-aconitate converted/min/mg protein.

2.11. Animal models of cardiac hypertrophy

Male Sprague-Dawley rats (8-week old, 200–250 g, Taconic Farm, Germantown, NY) received saline (25 μl/h) or AngII (200 ng/kg/min) by osmotic minipumps (ALZET model 2002) with and without NAC (200 mg/kg/d, i.p.) or losartan (50 mg/l in drinking water) for 2 weeks. At this concentration, the daily intake of losartan was approximately 10 mg/kg/d. Systolic blood pressure was measured non-invasively using the tail-cuff method (Life Science Instruments, Woodland Hills, CA). Left ventricular (LV) mass and body weight (BW) were measured for each condition before and after treatment.

2.12. Statistical analyses

All results are expressedas mean ± S.E.M. All data except time-related changes in blood pressure were compared by using one-way ANOVA and Fisher's exact test for post hoc analyses. A comparison of time-related changes in blood pressure among groups was performed using two-way ANOVA and Fisher's exact test for post hoc analyses. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell culture

Relatively pure (>95%) neonatal ventricular myocytes were confirmed by their morphological features using phase-contact microscopy and immunofluorescent staining with a monoclonal anti-cardiac MHC antibody (data not shown). There were no observable adverse effects of AngII, polyethylene glycol-superoxide dismutase (PEG-SOD), PEG-catalase, or other antioxidants at the concentrations used on cellular viability for all treatment conditions.

3.2. Antioxidants inhibit angiotensin II-induced cardiac myocyte hypertrophy

Treatment with AngII (1 μM) increased [^{3}H]-leucine uptake by 52 ± 5% and induced ANF promoter activity by 2.8 ± 0.4-fold ($P < 0.01$ for both compared to untreated condition) (Fig. 1). Lower concentrations of AngII (10 and 100 nM), however, did not increase [^{3}H]-leucine uptake (data not shown). AngII-induced [^{3}H]-leucine uptake was inhibited by AT1R blockade, losartan (10 μM), but not type 2 receptor blockade, PD123319 (10 μM). Co-treatment with *N*-acetylcysteine (NAC, 10 mM) or probucol (50 μM) inhibited AngII-induced increase in [^{3}H]-leucine uptake and ANF promoter activity by 94 ± 4% and 99 ± 5%, respectively. However, the antioxidants, vitamin C (500 μM) and glutathione (1 mM), have lesser inhibitory effects on AngII-induced [^{3}H]-leucine uptake and ANF promoter activity (41–45% and 26–53% inhibition, respectively, $P < 0.05$ for both compared to untreated condition) (Fig. 1). Since the generation of ROS have been associated with cardiac hypertrophy [4,7,13], we investigated whether superoxide anion (O_2^{-}) or hydrogen peroxide (H_2O_2) mediates AngII-induced cardiac hypertrophy. Co-treatment with cell-permeable PEG-SOD, but not PEG-catalase (except for minimal inhibition of ANF promoter activity) or PEG alone, inhibited AngII-induced [^{3}H]-leucine uptake and ANF promoter activity by 53 ± 6%

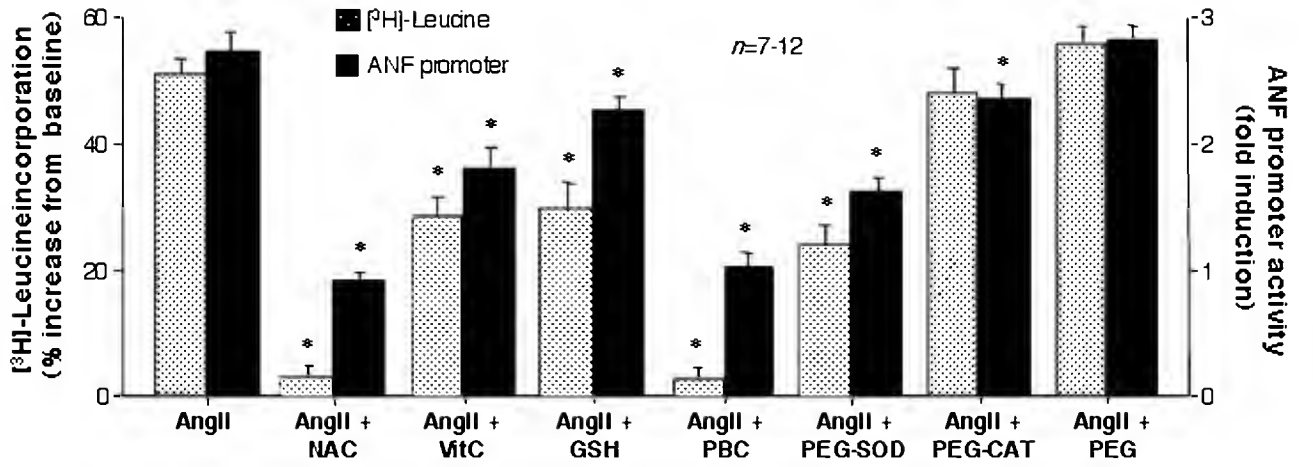

Fig. 1. Inhibition of cardiac myocyte hypertrophy by ATIR blocker and antioxidants. Effect of AngII (1 μM) with and without losartan (10 μM) or PD123319 (10 μM) on [^{3}H]-leucine incorporation (% increase from baseline). Effects of NAC 10 mM, vitamin C (VitC, 500 μM), reduced glutathione (GSH, 1 mM), probucol (PBC, 50 μM), PEG-conjugated SOD (PEG-SOD, 50 U/ml), PEG-catalase (500 U/ml), or PEG (0.32 mg/ml) on AngII-induced [^{3}H]-leucine incorporation (% increase from baseline) and ANF promoter activity (fold induction). Values are expressed as mean ± S.E.M., *$P < 0.05$ compared to AngII alone.

and 63 ± 5% ($P < 0.01$ for both compared to untreated condition). Treatment with antioxidants alone, however, did not affect basal [^{3}H]-leucine uptake or ANF promoter activity.

Treatment with AngII increased cell size by 25 ± 2%, which was completely blocked by co-treatment with NAC (92 ± 7% inhibition) (Fig. 2A). This corrected with cellular changes such as sarcomere organization. The hypertrophic process is also associated with the induction of fetal and structural cardiac genes, such as ANF and MLC-2v [14]. Indeed, the steady-state ANF and MLC-2v mRNA expressions were increased by about two fold following AngII stimulation ($n = 3$, $P < 0.01$) (Fig. 2B,C). Co-treatment with NAC inhibited AngII-induced ANF and MLC-2v mRNA expression in cardiac myocytes to below basal and basal levels, respectively. The relatively high basal expression of ANF and MLC-2v is probably attributed to the use of neonatal cardiac myocytes instead of adult cardiac cells. Nevertheless, these findings correlate with the inhibition of cardiac myocyte hypertrophy by NAC.

3.3. Antioxidants inhibit AngII-induced NAD(P)H oxidase activity and O_2^-· production

To determine whether the inhibitory effects of NAC on cardiac hypertrophy involve the inhibition of O_2^-· production, we monitored O_2^-· released into the supernatant by cardiac myocytes using ferricytochrome *c* reduction assay [11] or NAD(P)H-dependent oxidase activity in cell lysates with lucigenin chemiluminescence [10]. AngII increased O_2^-· production by 2.5-fold and NADPH-dependent oxidase activity by twofold (Fig. 3A). These increases in O_2^-· production and NADPH oxidase activity were completely blocked by co-treatment with NAC. AngII also increased NADH-dependent oxidase activity by 1.5-fold, which was also blocked by NAC (control, 6.9 ± 0.8; AngII, 10.3 ± 0.7; AngII + NAC, 7.5 ± 0.8 RLU/min). Indeed, PEG-SOD (50 U/ml), but not with PEG-catalase (500 U/ml), completely blocked AngII-induced O_2^-· production [8].

An important function of the small GTP-binding protein, Rac, in neutrophils, fibroblasts, and vascular smooth muscle cells is facilitating the assembly of NADPH oxidase, which is a major source of O_2^-· production in these cells, particularly in response to cytokines and AngII. Treatment with AngII increased membrane-associated RhoA and Rac1 GTP-binding activity by 2.5- to 3-fold, respectively ($P < 0.05$ for both) (Fig. 3B). Co-treatment with NAC, however, had no inhibitory effect on AngII-induced increases in membrane-associated RhoA and Rac1 GTP-binding activities. These findings indicate that the molecular target of intracellular oxidative stress is most likely a downstream target of Rac1.

3.4. Dominant-negative Rac1 inhibit AngII-induced myocardial oxidation and ANF promoter activity

To assess whether Rac1 mediates AngII-induced cardiac hypertrophy, we transfected c-myc-tagged dominant-negative Rac1 (N17Rac1) and performed dual fluorescence for c-myc and intracellular oxidation. Treatment with AngII increased intracellular oxidation, as determined by DCF fluorescence, in mostly cells that are not transfected with N17Rac1 (i.e. c-myc tag). Cells that are transfected with N17Rac1 exhibit decreased AngII-induced intracellular oxidation (Fig. 4A). Conversely, cells transfected with constitutively-active L61Rac1 showed enhanced intracellular oxidation (data not shown). In rat cardiac myocytes transfected with the empty vector (control), AngII increased ANF promoter activity by 2.8-fold (Fig. 4B). However, AngII-stimulated ANF promoter activity was completely blocked in rat cardiac myocytes transfected with N17Rac1. These results suggest that Rac1 mediates AngII-induced intracellular oxidation and hypertrophy.

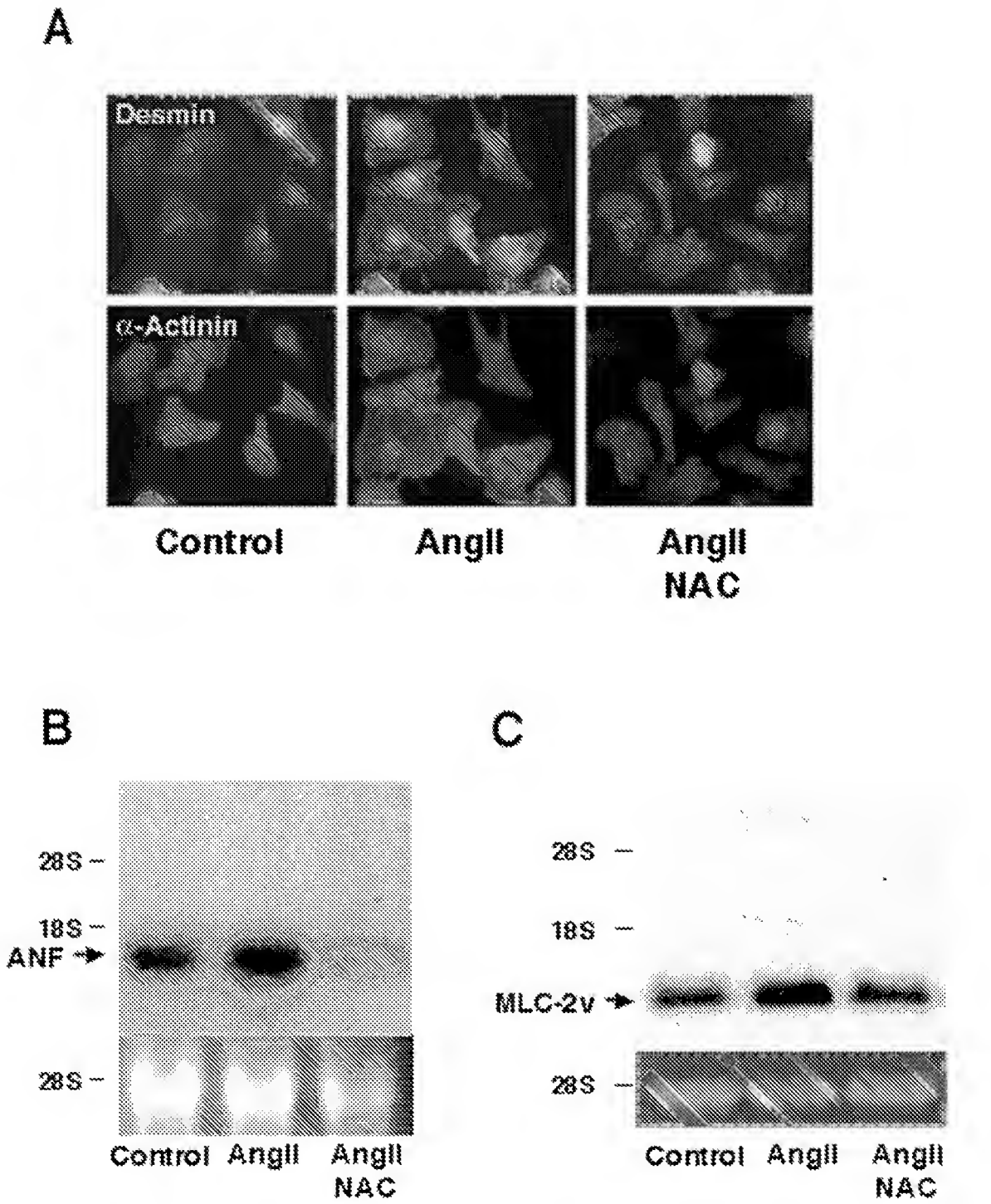

Fig. 2. Inhibition of cardiac sarcomere organization, myocyte size, and fetal gene expression by antioxidants. (A) Effects of AngII with and without NAC (10 mM) on cardiac myocyte size and sarcomere organization. Double immunofluorescent microscopy was performed to using specific antibodies to desmin (upper panel, green color) and α-actinin (lower panel, red color). Experiments were performed three times with similar results. Effect of AngII with or without NAC (10 mM) on steady state. (B) ANF and (C) MLC-2v mRNA expression at 24 h. Corresponding ethidium bromide-stained 28S ribosomal RNA was used to standardize loading. The results shown are representative of three separate experiments.

3.5. Antioxidants inhibit AngII-induced myocardial oxidation

To confirm that the effects of AngII and NAC on O_2^{-}· production corresponded to changes in intracellular oxidative stress, we assessed total intracellular oxidation in rat cardiac myocytes by DCF fluorescence. Stimulation with AngII produced a 2.8-fold increase in intracellular oxidative stress as measured by DCF fluorescence ($n = 3$, $P < 0.05$). This effect of AngII was inhibited by AT1R blocker, losartan (10 μM), but not type 2 receptor blocker, PD123319 (10 μM).

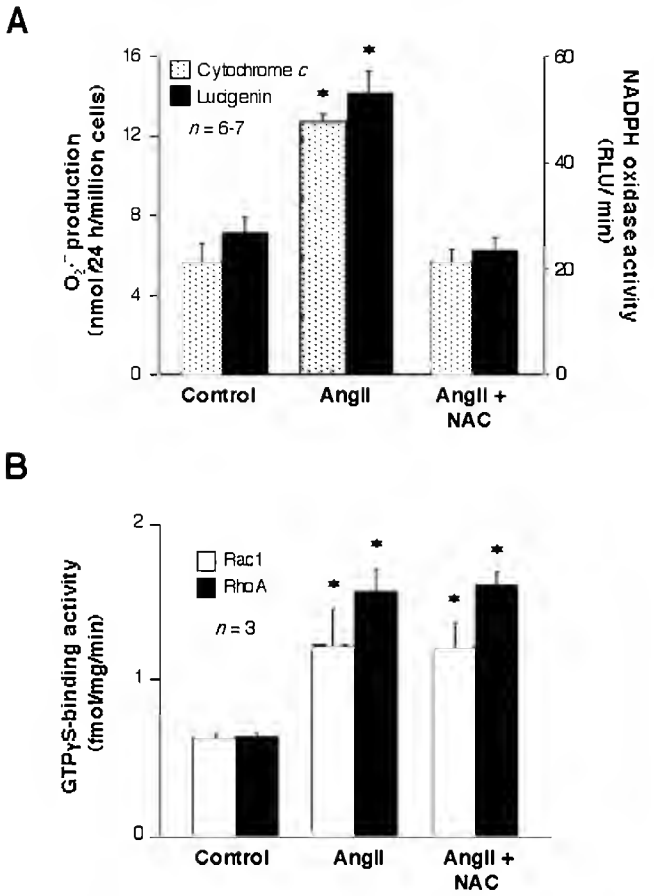

Fig. 3. Inhibition of superoxide anion production by antioxidants. (A) Effects of NAC (10 mM) on AngII-induced O_2^-· production and NADPH oxidase activity in rat cardiac myocytes as measured by ferricytochrome *c* reduction and lucigenin chemiluminescence, respectively. Values are expressed as mean ± S.E.M., $^*P < 0.01$ compared to unstimulated cells (control). (B) Effects AngII (1 μM) with and without NAC (10 mM) on membrane-associated Rac1 and RhoA GTP-binding activities in rat cardiac myocytes. Values are expressed as mean ± S.E.M., $^*P < 0.05$ compared to unstimulated cells (control).

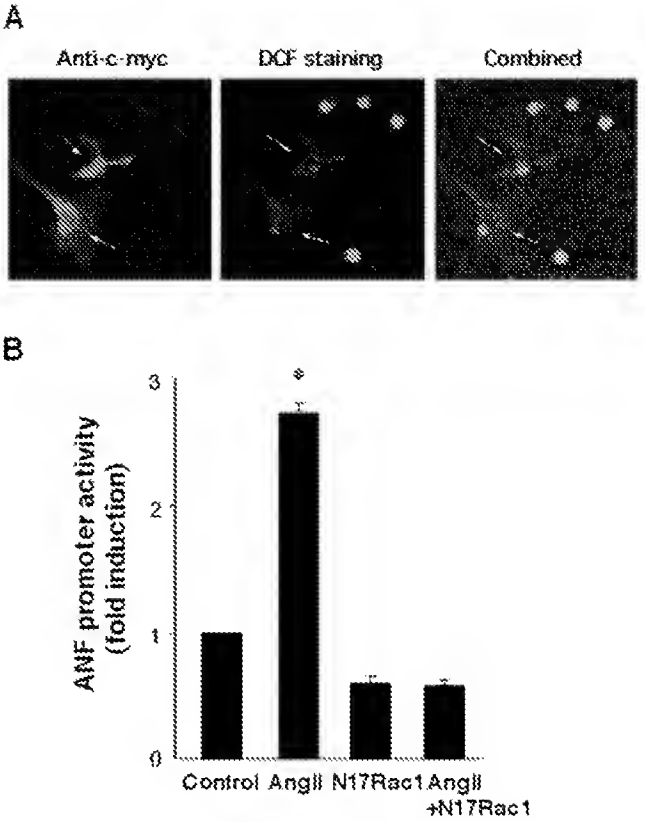

Fig. 4. Effect of dominant-negative Rac1 (N17Rac1) on AngII-induced (A) myocardial oxidation and (B) ANF promoter activity. Rat cardiac myocytes were transfected with N17Rac1 and stimulated with AngII (1 μM). Double fluorescent microscopy was performed to determine the expression of c-myc-tagged N17Rac1 in transfected cells (left panel, red color), corresponding levels of intracellular oxidation by DCF fluorescence (middle panel, green color), and dual immunofluorescence (right panel, both colors). Experiments were performed three times with similar results. (B) For AngII-induced ANF promoter activity, values are expressed as mean ± S.E.M., $^*P < 0.01$ compared with transfection with vector alone (control).

AngII-induced DCF fluorescence was completely blocked by the addition of PEG-catalase which converts H_2O_2 to H_2O, but not by PEG-SOD, which enhances rather than inhibits the conversion of O_2^-· to H_2O_2 (Fig. 5). PEG alone had no effect on AngII-induced intracellular oxidation. This increase was also completely blocked by co-treatment with NAC, indicating that under the treatment condition, NAC inhibited AngII-induced intracellular oxidative stress.

3.6. Antioxidants inhibit cardiac O_2^- production and hypertrophy in vivo

To determine whether our in vitro findings have physiological relevance, we evaluated the effects of NAC using a widely accepted model of cardiac hypertrophy [15]. In rats, AngII infusion (200 ng/kg/d, 14 d) caused a substantial increase in systolic blood pressure (118 ± 6 to 180 ± 5 mmHg, $P < 0.01$, $n = 5$), which was not affected by co-treatment with NAC (182 ± 3 mmHg, $P > 0.05$, $n = 8$). Furthermore, treatment with NAC alone had no effect on basal systemic blood pressure, BW or LV mass. Co-treatment of losartan (10 mg/kg/d, 14 d) completely blocked AngII-induced BP increase (119 ± 3 mmHg, $n = 5$). AngII increased O_2^-· production by 40% (42 ± 4 to 59 ± 5 nmol/mg, $n = 6$, $P < 0.05$) in intact, non-homogenized, rat heart tissues; an effect which was completely blocked by co-treatment with NAC (42 ± 6 nmol/mg, $n = 8$, $P < 0.05$ compared to AngII infusion) (Fig. 6A). Similarly, AngII increased NADPH-dependent oxidase activity (i.e. lucigenin chemiluminescence) by 43%, which was also completely blocked by NAC. These results correlated inversely with a 41% decrease in aconitase activity in rat heart tissues after AngII infusion (440 ± 27 to 260 ± 41 nmol/min/mg, $n = 6$, $P < 0.005$), which was completely restored by co-treatment with losartan or NAC (446 ± 29 and 450 ± 25 nmol/min/mg, respectively, $n = 6$, $P < 0.001$ compared to AngII infusion) (Fig. 6B).

In hearts from rats treated with AngII infusion, there were increases in membrane-associated RhoA and Rac1 GTP-binding activities, both of which were unaffected by co-treatment with NAC (Fig. 7A). These findings, therefore,

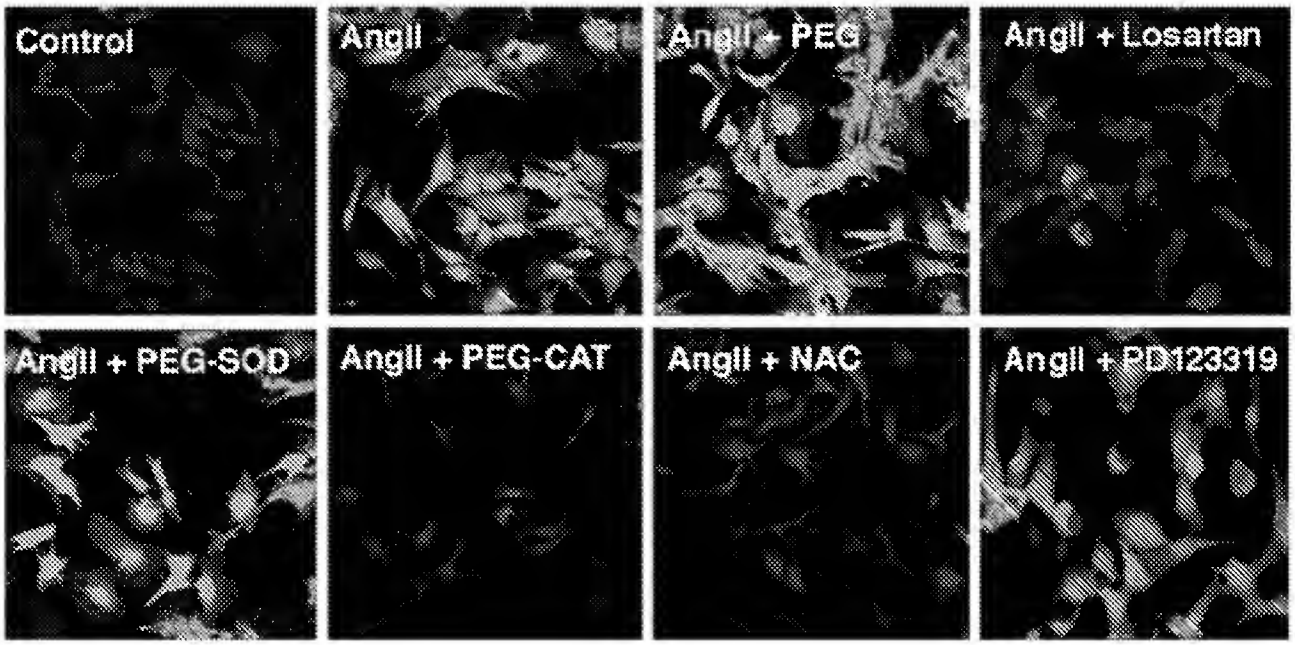

Fig. 5. Inhibition of intracellular oxidation by antioxidants. Effects of losartan (10 μM), PD123319 (10 μM), PEG (0.32 mg/ml), PEG-SOD (50 U/ml), PEG-catalase (500 U/ml), or NAC (10 mM) on AngII-induced intracellular oxidation (DCF fluorescence). Results shown were chosen from five random fields and are representative of three separate experiments.

suggest that inhibition of Rho proteins does not contribute to NAC's inhibitory effect on AngII-induced $O_2^-\cdot$ production in vivo AngII infusion caused a significant increase in LV mass and LV to BW ratio (Fig. 7B). The increases in LV mass and LV/BW ratio by AngII were completely inhibited by co-treatment with NAC or losartan. However, under the short duration of our experimental conditions, AngII did not cause cardiac fibrosis (data not shown).

4. Discussion

We have shown that various antioxidants, such as NAC and probucol, and to a lesser extent, vitamin C and reduced glutathione, can inhibit AngII-induced [^{3}H]-leucine incorporation and ANF promoter activity via inhibition of myocardial $O_2^-\cdot$ generation. Stimulation with AngII increased protein synthesis, sarcomere organization, and the re-expression of embryonic genes, such as ANF and MLC-2v, in rat cardiac cardiomyocytes. These effects were associated with AngII-induced increase in lucigenin chemiluminescence, DCF fluorescence, and $O_2^-\cdot$ production. The hypertrophic effect of AngII is mediated by $O_2^-\cdot$-derived ROS because the $O_2^-\cdot$ scavenger, SOD, and the nonspecific antioxidant, NAC or probucol, completely inhibited AngII-induced cardiac myocyte [^{3}H]-leucine incorporation and ANF promoter activity, re-expression of embryonic genes and $O_2^-\cdot$ production in rat cardiac myocytes. The anti-hypertrophic effects of antioxidants were observed both in vitro and in vivo and occurred independent of effects on Rho and Rac1. These findings, therefore, suggest that oxidant-induced hypertrophy occurs downstream of Rho and Rac1 signaling and that antioxidant therapy may be useful in preventing the development of cardiac hypertrophy. Although previous studies have shown that antioxidants in general could attenuate cardiac hypertrophy, the precise ROS, which mediates the hypertrophic response has not been well characterized [16,17]. The findings of this study suggest that it is $O_2^-\cdot$, but not H_2O_2, which mediates AngII-induced [^{3}H]-leucine incorporation and ANF promoter activity in vitro and cardiac hypertrophy in vivo.

The expression of AT1R, which mediates the increase in ROS, is elevated in cardiovascular tissue from animals with hypertension and LV hypertrophy [18,19]. In contrast, other studies suggest that AT1R may not be necessary for the hypertrophic response to pressure overload [20]. Nevertheless, cardiac hypertrophy caused by AngII infusion occurs in part, through the formation of ROS, and angiotensin converting enzyme (ACE) inhibitors or AT1R blockade has some beneficial effects in the formation of cardiac hypertrophy and remodeling [8,21,22]. AngII stimulates $O_2^-\cdot$ production through NADPH oxidase activation [23,24]. Indeed, in contrast to wild-type mice, gp91^{PHOX}- or Nox2-deficient mice do not develop cardiac hypertrophy or fibrosis in response to AngII treatment [25]. The upstream signaling pathway leading to ROS-dependent hypertrophic response may occur through the activation of the heterotrimeric G-proteins (i.e. G_q) and small G-proteins (i.e. Rac1 and RhoA) [26–29]. In particular, Rac1 is an important mediator of ROS production in both vascular smooth muscle and cardiomyocytes.

In summary, our findings suggest that increased ROS may be the common underlying mechanism by which high circulating or tissue levels of AngII lead to the development of cardiac hypertrophy. Our data indicate that AngII causes

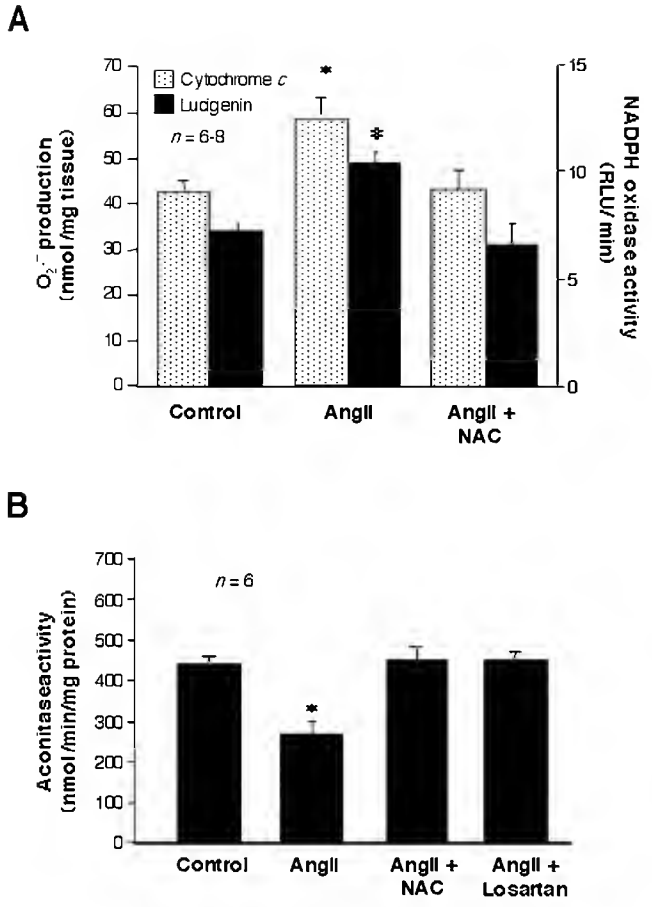

Fig. 6. Inhibition of superoxide anion production in rat hearts by antioxidants. Effects of AngII (200 ng/kg/min) infusion with and without NAC (200 mg/kg/d, 14 d) or losartan (10 mg/kg/d, 14 d) on (A) NADPH oxidase activity (lucigenin chemiluminescence) and O_2^- released from intact hearts (ferricytochrome *c* reduction) and (B) myocardial aconitase activity. Values are expressed as mean ± S.E.M., $^*P < 0.05$ compared to vehicle-treated animals (control).

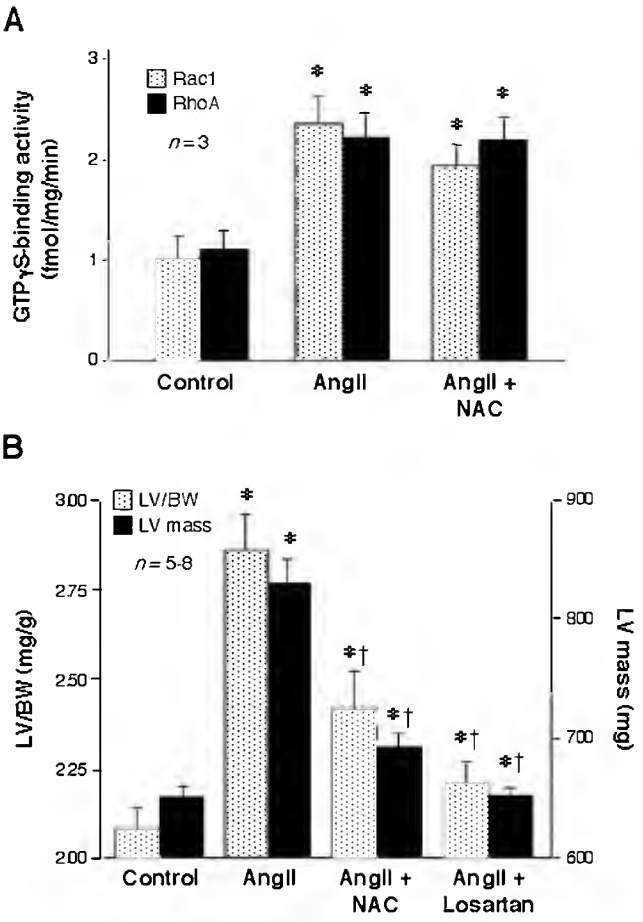

Fig. 7. Inhibition of cardiac hypertrophy by antioxidants. Effects of AngII (200 ng/kg/min) infusion with and without NAC (200 mg/kg/d, 14 d) or losartan (10 mg/kg/d, 14 d) on (A) membrane-associated myocardial Rac1 and RhoA GTP-binding activities and (B) LV/BW ratio and LV mass in rats. Values are expressed as mean ± S.E.M., $^*P < 0.01$ compared to vehicle-treated sham-operated animals (control), $^\dagger P < 0.05$ compared to AngII-treated animals.

cardiac hypertrophy via the generation of ROS, and that these responses are inhibited by treatment with antioxidants. These findings, therefore, suggest that antioxidant therapy may be an effective treatment strategy for attenuating the hypertrophic process, regardless of the etiology. However, supporting data from large clinical trials with antioxidants are needed before such recommendations can be made.

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